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High Serum Level of Antithymocyte Globulin Immediately before Graft Infusion Is Associated with a Low Likelihood of Chronic, But Not Acute, Graft-versus-Host Disease

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A B S T R A C T

Rabbit antithymocyte globulin (ATG) is administered during transplant conditioning to decrease the risk of both acute graft-versus-host disease (aGVHD) and chronic graft-versus-host disease (cGVHD). Here we evaluated the relationship between the serum concentration of ATG (capable of binding to lymphocytes) immediately before graft infusion (day 0) or on day +7 or +28 post-transplantation and the development of aGVHD or cGVHD. We studied 180 patients whose conditioning included 4.5 mg/kg antithymocyte globulin (ATG; Thymoglobulin). For aGVHD, we found no association with ATG levels on day 0. Nevertheless, high day +7 and +28 ATG levels were associated with a low likelihood of aGVHD. For cGVHD, high ATG levels at all 3 time points (days 0, +7, and +28) were associated with a low likelihood of cGVHD. In conclusion, high-dose ATG administration at the time of graft infusion appears to inhibit the development of cGVHD, but not aGVHD; however, higher ATG levels on days +7 and +28 are associated with lower rates of both aGVHD and cGVHD.

INTRODUCTION

The addition of rabbit antithymocyte globulin (ATG) to pretransplantation conditioning has been shown to decrease the incidence of acute graft-versus-host disease (aGVHD) and chronic graft-versus-host disease (cGVHD) in recipients of both unrelated and related donor grafts [1-11]. Two brands of rabbit ATG are available: Thymoglobulin (Genzyme/Sanofi, Cambridge, MA), produced from sera of rabbits immunized with human thymocytes, and ATG-F (Fresenius, Bad Homburg, Germany), produced from sera of rabbits immunized with Jurkat T cells. In both cases, ATG is produced by purification of IgG from the immune sera.

ATG is polyclonal and contains antibodies against antigens expressed on lymphocytes, other leukocytes, and nonleukocytes [12-15]. It is generally believed that the fraction of antibodies within ATG that can bind to lymphocytes is the functional (ie, anti-GVHD) fraction. Consistent with this idea, we have reported that high serum levels of this fraction on days +7 and +28 were associated with a low likelihood of both aGVHD and cGVHD [16]. We hypothesized that day 0 levels may be even more strongly associated with GVHD than day +7/+28 levels, because the day 0 levels should not be influenced by the variability in ATG elimination between day 0 and day +7/+28. The variability in elimination could be related to, for example, differences in graft content of cells that can adsorb ATG (on target antigens or Fc receptors) or differences in the number of recipient cells that can adsorb or eliminate ATG.

The primary objective of the present study was to evaluate the associations between the day 0, +7, and +28 ATG levels and aGVHD/cGVHD. The secondary objective was to evaluate associations between the day 0, +7, and +28 levels and relapse, cytomegalovirus (CMV) reactivation, post-transplantation lymphoproliferative disorder (PTLD), and death.

PATIENTS AND METHODS

Patients and Transplantation

We studied 185 consecutive recipients of allogeneic filgrastim-mobilized peripheral blood stem cells (PBSCs) for a hematologic malignancy who consented to participate in the study. All patients received ATG (Thymoglobulin) as part of pretransplantation conditioning. All transplantations were performed between December 2008 and December 2012. Patients who failed to engraft (n = 3), relapsed (n = 0), or died (n = 2) before day +30 were excluded; their ATG levels on days 0, +7 and +28 were not markedly different compared with the 180 patients included in the study, data for whom we report here. For 177 of these 180 patients, conditioning consisted of fludarabine 250 mg/m^2, busulfan ~12.8 mg/kg (pharmacokinetically adjusted), and ATG 0.5 mg/kg on day -2, 2.0 mg/kg on day -1, and 2.0 mg/kg on day 0 (total, 4.5 mg/kg) [7]. The last dose of ATG (day 0 dose) was given before graft infusion. Total body irradiation (TBI; 4 cGy) was also administered to 133 patients [17]. Table 1 shows patient and donor characteristics.

Post-transplantation GVHD prophylaxis consisted of cyclosporine from day -1 up to 3 months post-transplantation, at a starting dose of 2.5 mg/kg every 12 hours and later targeting trough serum levels of 200 to 400 g/L and methotrexate on days +1, +3, +6, and +11 (first dose, 15 mg/m^2 IV; next 3 doses, 10 mg/m^2 IV; the last dose was omitted in rare patients with airway-jeopardizing mucositis) [7]. No antifungal prophylaxis was given routinely until 2010; thereafter, fluconazole was given on days 0 to +28. Routine antibacterial prophylaxis was not provided, except for trimethoprim-sulfamethoxazole for pneumocystis prophylaxis given up to 6 months post-transplantation or longer in cases of cGVHD requiring systemic therapy. Viral prophylaxis consisted of acyclovir or valacyclovir for up to 2 years

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Table 1
Patient and Donor Characteristics

Characteristic	Value
Total patients, n	180
Patients with day 0 serum samples, n	152
Patients with day 7 serum samples, n	164
Patients with day 28 serum samples, n	160
Patient age at transplantation, yr, median (range)	50 (18-66)
Donor age at transplantation, yr, median (range)	36 (13-68)
Patient sex, M/F, n	104/76
Donor sex, M/F, n	116/64
Diagnosis, n	
Acute lymphoblastic leukemia	35
Acute myelogenous leukemia	68
Chronic lymphocytic leukemia	15
Chronic myelogenous leukemia	7
Myelodysplastic syndrome	21
Non-Hodgkin lymphoma	13
Myelofibrosis	10
Other hematologic malignancies	9
Disease risk stage, n*	
Good	94
Poor	86
Donor type, n	
HLA-matched sibling	67
Others	113
HLA match (-A, -B, -C, -DRB1, and -DQB1), n	
10/10 allele-matched	153
8-9/10 allele-matched	27
Conditioning regimen, n	
Flu + Bu + ATG + TBI	131
Flu + Bu + ATG	46
Flu + Cy + ATG + TBI	2
Cy + Bu + ATG	1
Acute GVHD† grade, n	
Grade 0	81
Grade I	51
Grade II	26
Grade III	15
Grade III-IV	1
Grade IV	5
Median day of onset of grade II-IV aGVHD (range)	56 (15-99)
Chronic GVHD,‡ n	
None	84
NNST	20
NST	48
Not applicable§	28
Median onset day of chronic GVHD NST (range)	126 (89-446)
Number of patients with CMV reactivation above threshold for preemptive therapy	45
Median day of onset of CMV reactivation (range)	43.5 (20-175)
Number of patients with PTLD	15
Median day of onset of PTLD (range)	55.5 (33-408)
Number of patients with relapse	33
Median day of onset of relapse (range)	182 (39-946)
Median day of follow-up for death (range)‖	448 (31-1448)
Median day of follow-up for death in patients who did not die (range)	566 (68-1448)
Median day of follow-up for relapse and nonrelapse death (range)¶	422 (31-1448)
Median day of follow-up for relapse and nonrelapse death in patients who did not relapse or die (range)	547 (59-1448)
Median day of follow-up for GVHD, CMV reactivation and PTLD (range)**	387 (31-1448)
Median day of follow-up for GVHD, CMV reactivation and PTLD in patients who did not develop graft failure, relapse, secondary malignancy, or death	564 (59-1448)

Flu indicates fludarabine; Bu, busulfan; TBI, total body irradiation; Cy, cyclophosphamide.

* Good risk disease/stage includes primary acute leukemia in first remission, chronic myelogenous leukemia in first chronic phase, and myelodysplasia with <5% marrow blasts. All other diseases/disease stages were considered poor risk.

† aGVHD occurred in 66 of 113 (58.4%) unrelated transplant recipients and in 34 of 65 (52.3%) matched sibling transplant recipients ($P = .4297$, chi square test).

‡ cGVHD occurred in 40 of 100 (40%) unrelated transplant recipients and in 28 of 56 (50%) matched sibling transplant recipients ($P = .2269$, chi square test).

§ Patients who developed second malignancy, relapse, or death or were lost to follow-up before day 100.

‖ Patients were followed until death or until the last day known to be alive.

¶ Patients were followed until they developed relapse or death or were lost to follow-up.

** Patients were followed until they developed graft failure, second malignancy (including PTLD), relapse, or death, or until they were lost to follow-up (defined as the last day on which medically meaningful information was available).

post-transplantation or longer in cases of cGVHD requiring systemic therapy and a preemptive CMV strategy [18].

Measurement of ATG Levels

Blood was drawn from patients on day 0 (within 15 minutes before graft infusion), day +7 (range, day +6 to day +8), and day 28 (range, day +24 to day +36) post-transplantation. Serum was stored in tightly sealed vials at minus 80°C until ATG level determination. We measured ATG levels (capable of binding to lymphocytes) using the flow cytometry–based assay developed by Kakhniashvili et al. [19] with minor modifications [16]. In brief, standards of known ATG concentrations, ranging from 20 to 0.0098 mg/L were prepared by serial 2-fold dilution. Peripheral blood mononuclear cells from a healthy volunteer were incubated with patient serum or an ATG standard. The cells (coated with ATG) were then labeled with phycoerythrin-conjugated goat anti-rabbit IgG. After flow cytometry data acquisition, lymphocytes were gated by forward and side scatter characteristics. Phycoerythrin fluorescence was measured for each standard and for each patient serum sample included in the run. Plotting ATG levels of standards versus the median channel of phycoerythrin fluorescence generated a standard curve, from which patient ATG levels were then extrapolated.

Definitions of Outcomes

aGVHD and cGVHD were categorized according to National Institutes of Health (NIH) criteria [20]. A minority of patients with insufficient information in their charts for unequivocal categorization of post–day +100 GVHD as cGVHD versus late aGVHD were classified as having cGVHD. aGVHD was graded in accordance with the 1994 consensus conference [21]. Chronic GVHD was graded as none, not needing systemic therapy (NNST), or needing systemic therapy (NST). "Any cGVHD" refers to cGVHD NNST or NST. Relapse, death, and nonrelapse death were defined using standard criteria. CMV reactivation was defined as CMV DNAemia above our institutional threshold for preemptive antiviral therapy (25,000 IU/mL plasma) or CMV disease. PTLD was defined as an illness with signs or imaging results consistent with PTLD (eg, fever not due to other causes, lymphadenopathy, splenomegaly, mass) with Epstein-Barr virus (EBV) DNAemia >400 copies/μg of leukocyte DNA or >40,000 copies/mL whole blood or histological evidence of PTLD, including in situ hybridization for EBV-encoded RNA.

Statistics

ATG levels in patients with versus patients without grade II-IV aGVHD, grade III-IV aGVHD, any cGVHD, cGVHD NST, relapse, death, nonrelapse death, CMV reactivation, and PTLD were compared using the Mann-Whitney-Wilcoxon (MWW) test. For outcomes for which ATG levels appeared to differ significantly between patients with and those without the outcome ($P \leq .05$, MWW test), we proceeded to multivariate analysis. Using log-binomial regression models, we determined whether patients with ATG levels above the cutoff had a higher/lower likelihood of the outcome compared with patients with ATG levels below the cutoff (multivariate analysis adjusting for confounding factors known to be associated with the outcome). The cutoff ATG levels were determined using the receiver operating characteristic (ROC) curve as the cutoff associated with the highest sum of sensitivity and specificity. To avoid having too small patient groups

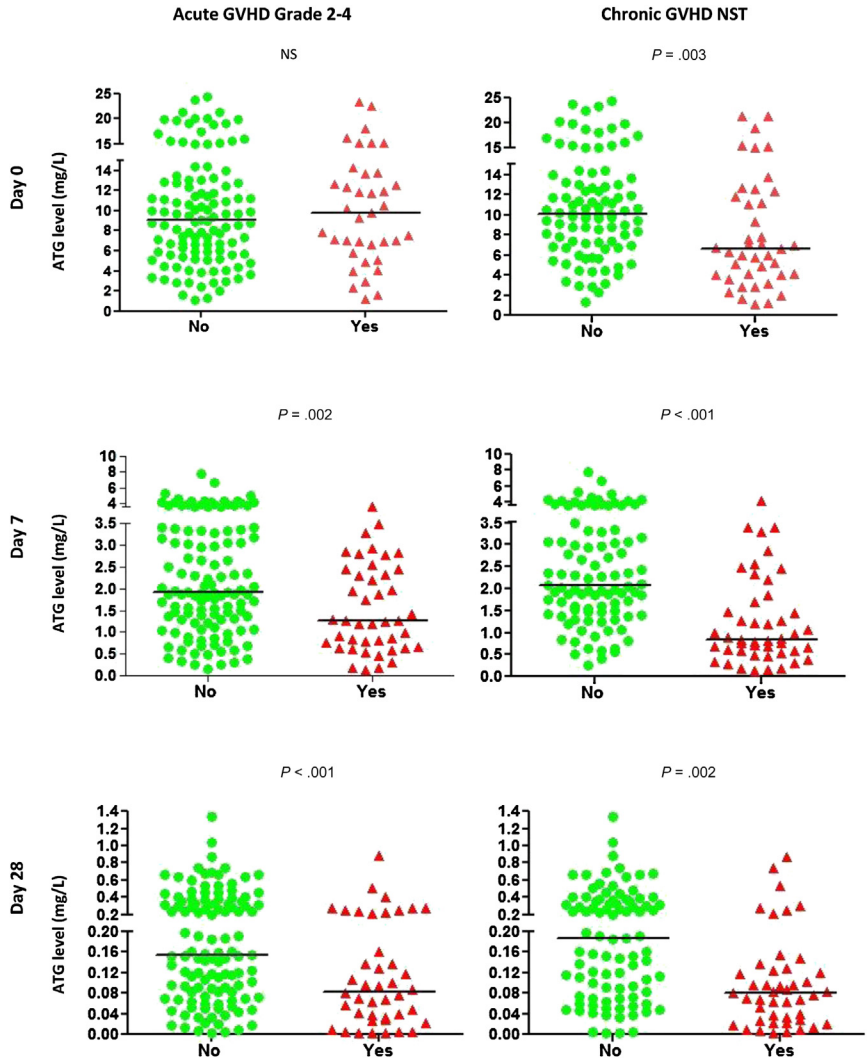

Figure 1. Association (or lack of association) between ATG level and GVHD in univariate analysis. Horizontal bars indicate median values. *P* values shown are from the MWW rank-sum test. NS, not significant; NST, needing systemic therapy.

with ATG levels above/below the cutoff, only cutoffs between the 10th and 90th percentiles were considered. Confounding factors (covariates) considered in the multivariate analyses for aGVHD and cGVHD were recipient age (above versus below 45 years), donor age (above versus below 45 years), donor type (HLA-matched sibling versus other), and donor/recipient sex match (M/M versus other). For CMV reactivation, we considered donor/recipient CMV serostatus (−/−, −/+, +/+, and +/−), presence of significant GVHD (either grade II-IV aGVHD or cGVHD NST) before CMV reactivation (yes versus no), and donor type (HLA-matched sibling versus other). For PTLD, we considered donor/recipient EBV serostatus (+/+ versus other, including unknown), presence of significant GVHD (either grade II-IV aGVHD or cGVHD NST) before PTLD onset (yes versus no), donor type (HLA-matched sibling versus other), and recipient age (above versus below 45 years) [22,23].

Batch of ATG was not included as a covariate in any of the multivariate analyses, because batch-to-batch variability in ATG potency has been shown to be minimal [24,25]. In cases of nonconvergence of log-binomial regression, Poisson regression with Huber-White sandwich robust variance [26] was used to approximate the log-binomial model.

Because the ATG levels were skewed toward lower levels, we also ran all multivariate analyses using log-transformed ATG levels. The results were virtually identical to those obtained using raw ATG levels; thus, only the results obtained with the raw ATG levels are shown.

MWW test analyses were performed using Prism software (GraphPad Software, La Jolla, CA). ROC curve analysis and Spearman's rank correlation test were performed using MedCalc software (MedCalc, Mariakerke, Belgium). All multivariate analyses were performed using Stata software (StataCorp, College Station, TX).

RESULTS

GVHD

ATG levels on day 0

Univariate analysis revealed no significant association between ATG levels on day 0 and the development of aGVHD grade II-IV or aGVHD grade III-IV (Figure 1 and Table 2). However, patients who developed any cGVHD had lower ATG levels compared with those who did not develop cGVHD ($P < .001$), and patients who developed cGVHD NST had lower ATG levels compared with those who did not develop cGVHD or had cGVHD NNST ($P = .003$). The association between day 0 ATG level and cGVHD was also significant in multivariate analyses (Table 2). Patients with an ATG level >8.12 mg/L on day 0 had a 0.5-fold risk of developing any cGVHD ($P < .001$) compared with those with an ATG level <8.12 mg/L. Similarly, patients with an ATG level >7.83 mg/L on day 0 had a 0.4-fold risk of developing cGVHD NST ($P < .001$) compared with those with an ATG level <7.83 mg/L.

ATG level on day +7

As shown in Figure 1 and Table 2, in both univariate and multivariate analyses, there was a significant association between low day +7 ATG level and either aGVHD or cGVHD.

Table 2
Association (or Lack of Association) between ATG Level and GVHD

	Acute GVHD Grade II-IV		Acute GVHDGrade III-IV		Any Chronic GVHD, NNST or NST		Chronic GVHD, NST	
	Yes	No	Yes	No	Yes	No	Yes	No
ATG levels on day								
Median ATG levels	9.788	9.049	11.780	9.413	6.977	10.590	6.589	10.080
P value*	.770		.778		<.001		.003	
Cutoff ATG level					>8.12		>7.83	
Adjusted relative risk (95% CI)					0.5 (0.3-0.7)		0.4 (0.2-0.6)	
Adjusted *P* value†					<.001		<.001	
ATG levels on day 7								
Median ATG level	1.255	1.915	1.078	1.907	1.126	2.051	0.846	2.067
P value*	.002		.005		<.001		<.001	
Cutoff ATG level	>1.29		>1.41		>1.26		>1.26	
Adjusted relative risk (95% CI)	0.3 (0.2-0.6)		0.3 (0.1-0.8)		0.4 (0.2-0.5)		0.2 (0.1-0.3)	
Adjusted *P* value†	<.001		.012		<.001		<.001	
ATG levels on day 28								
Median ATG level	0.084	0.153	0.049	0.152	0.086	0.187	0.081	0.186
P value*	<.001		<.001		.002		<.001	
Cutoff ATG level	>0.14		> 0.08		>0.14		>0.14	
Adjusted relative risk (95% CI)	0.4 (0.2-0.8)		0.2 (0.1-0.6)		0.6 (0.4-0.9)		0.3 (0.2-0.6)	
Adjusted *P* value†	.005		.006		.008		.001	

* Mann-Whitney-Wilcoxon test (univariate analysis).
† Log-binomial regression (multivariate analysis).

ATG level on day +28

Similar to day +7, in both univariate and multivariate analyses, there was a significant association between low day +28 ATG level and both aGVHD and cGVHD (Figure 1 and Table 2).

Outcomes Other Than GVHD

No significant associations were found between ATG level on day 0, day +7, or day +28 and relapse, death, or nonrelapse death (Table 3). For CMV reactivation, there was no significant association with ATG level on day 0 or day +7. There appeared to be an association between low day 28 ATG level and CMV reactivation in univariate analysis, but not in multivariate analysis (Table 3). For PTLD, there was no significant association with ATG level on day 0; however, consistent with our previous observation [16], significant associations between high day +7 and day +28 ATG level and PTLD were noted in both univariate and multivariate analyses (Table 3).

Decrease in ATG Level and GVHD

Given the association between day +7 and day +28, but not day 0 ATG level and aGVHD, we hypothesized that aGVHD may be associated with the decrease in ATG level from day 0 to day +7 or +28 (which depends on the rate of ATG elimination from serum), rather than with the level on day 0. Consistent with this hypothesis, a high relative decrease in ATG level from day 0 to day +7 was associated with subsequent aGVHD in both univariate and multivariate analyses (Table 4). Analogously, a high relative decrease in ATG level from day 0 to day +28 also was associated with aGVHD (Table 4). For cGVHD, we found no significant

Table 3
Association (or Lack of Association) between ATG Levels and Clinical Outcomes Other Than GVHD

Variable	Relapse		Death		Nonrelapse Death		CMV Reactivation*		PTLD	
	Yes	No	Yes	No	Yes	No	Yes	No	Yes	No
ATG level on day 0										
Median ATG level	9.003	9.401	8.291	9.359	9.187	9.206	10.430	8.815	11.480	8.909
P value†	.596		.471		.679		.509		.222	
Cutoff ATG level										
Adjusted relative risk (95% CI)										
Adjusted *P* value‡										
ATG level on day 7										
Median ATG level	2.031	1.789	2.030	1.852	1.734	1.869	1.734	1.875	3.116	1.746
P value†	.129		.660		.500		.406		.012	
Cutoff ATG level									>1.87	
Adjusted relative risk (95% CI)									5.1 (1.5-17.5)	
Adjusted *P* value‡									. 010	
ATG level on day 28										
Median ATG level	0.152	0.127	0.112	0.140	0.084	0.138	0.090	0.152	0.331	0.120
P value†	.300		.647		.219		.024		<. 001	
Cutoff ATG level							>0.10		>0.15	
Adjusted relative risk (95% CI)							0.7 (0.4-1.2)		9.7 (2.3-42.9)	
Adjusted *P* value‡							.192		.002	

* CMV reactivation above threshold for preemptive therapy.
† MWW test (univariate analysis).
‡ Log-binomial regression (multivariate analysis).

Table 4
Association (or Lack of Association) between Relative Decrease in ATG Level and GVHD

	Acute GVHD*		Chronic GVHD*	
	Yes	No	Yes	No
Relative decrease in ATG level from day 0 to day 7†				
Median ATG drop, %	82.19	78.93	82.59	78.04
P value‡	.005		.129	
Cutoff ATG drop, %	>87.85			
Adjusted relative risk (95% CI)	2.7 (1.5-4.9)			
Adjusted *P* value§	.001			
Relative decrease in ATG level from day 0 to day 28¶				
Median ATG drop, %	99.10	97.89	98.93	98.27
P value‡	.011		.245	
Cutoff ATG drop, %	>98.56			
Adjusted relative risk (95% CI)	2.6 (1.4-4.9)			
Adjusted *P* value§	.002			

* Acute GVHD indicates acute GVHD grade II-IV; chronic GVHD indicates any chronic GVHD.
† Percentage decrease in ATG level from day 0 to day 7.
‡ Mann-Whitney-Wilcoxon test (univariate analysis).
§ Log-binomial regression (multivariate analysis).
¶ Relative decrease indicates the percentage decrease in ATG level from day 0 to day 28.

association with a relative decrease in ATG level from day 0 to day +7 or from day 0 to day +28 (Table 4).

We also estimated the clearance and half-life of ATG in patients in whom sera were available at all 3 time points (days 0, +7, and +28) (n = 130), and determined whether the clearance was associated with GVHD. ATG clearance was estimated using the formula Cl = dose/AUC, where dose is the administered dose of ATG and AUC is the area under the serum concentration versus time curve [27]. The median ATG clearance was 0.023 L/kg/day (range, 0.005 to 0.26 L/kg/day), and the median half-life was 5 days (range, 2.2 to 16.2 days). There was a significant association between ATG clearance and aGVHD grade II-IV in both univariate analysis (median clearance in patients with aGVHD grade II-IV, 0.028 L/kg/day, in patients without aGVHD grade II-IV, 0.021 L/kg/day; $P = .028$, MWW test; Table 5) and multivariate analysis (patients with clearance >0.011 L/kg/day had a 9.4-fold increased likelihood of developing aGVHD grade II-IV compared with those with clearance <0.011 L/kg/day; $P = .025$). Similarly, patients who developed cGVHD had significantly higher clearance compared with those without cGVHD in both univariate and multivariate analyses (Table 5).

Decrease in ATG Level and Immune Cell Subset Counts

We also evaluated whether the decrease in ATG level (capable of binding to lymphocytes) from day 0 to day +28 was related to lymphocyte subset counts. In 32 patients, we measured blood counts of T, B, and natural killer cells on day +28; the counts were too low to measure on days 0 and +7 [28]. There was a significant correlation between the relative decrease in ATG level from day 0 to day +28 and the day +28 T cell count (Table 6).

We then evaluated possible associations between total WBC or lymphocyte count in transplant recipients and the decrease in ATG level. Day 0 WBC count was available for 123 patients. In 87 of these 123 patients, the WBC was <0.5/nL, and thus, in accordance with our hematology laboratory's policy, a differential blood count was not performed. In the remaining 35 patients, the WBCs were composed only of neutrophils and monocytes, with no lymphocytes, eosinophils, or basophils detected. There was no significant association between the relative decrease in ATG level from day 0 to day +7 (n = 35) and the day 0 WBC count (Spearman's correlation coefficient, $r = 0.215$; $P = .22$). Day +7 WBC count was <0.5/nL in all patients; given the inaccuracy of measurement of WBC <0.5/nL, we did not evaluate for a possible association between day +7 WBC count and decrease in ATG level. On day +28, WBC count was consistently >0.5/nL. There was no significant association between the relative decrease in ATG level from day 0 to day +28 (n = 107) and WBC count on day 28 ($r = 0.035$; $P = .72$). Similarly, no significant association was found between the relative decrease in ATG level from day 0 to day +28 (n = 107) and lymphocyte count on day +28 ($r = 0.133$; $P = .173$).

We next evaluated for a possible association between graft WBC or mononuclear cell (MNC) count and decreased ATG level in 110 patients. There was no significant association between the relative decrease in ATG level from day 0 to day +7 and total WBCs in grafts ($r = 0.048$; $P = .620$). Similarly, there also was no significant association between the relative decrease in ATG level from day 0 to day +7 and MNC count in grafts ($r = 0.147$; $P = .151$).

We then evaluated possible association between ATG clearance and recovery of WBCs or lymphocytes by day +28 (n = 101). We found no significant association between the clearance of ATG and total WBC count on day +28 ($r = -0.108$; $P = .280$). Similarly, there was no significant association between ATG clearance and total lymphocyte count on day +28 ($r = -0.043$; $P = .670$).

DISCUSSION

GVHD is a major obstacle to safe allogeneic transplantation. In the present study, we found that low serum levels of ATG on day 0 were associated with a high likelihood of cGVHD, but not aGVHD (either grade II-IV or grade III-IV). In contrast, low day +7 and +28 levels were associated with both aGVHD and cGVHD, consistent with our previous study [16]. In disagreement with our present results,

Table 5
Association (or Lack of Association) between ATG Clearance and GVHD

Variable	Acute GVHD Grade II-IV		Acute GVHD Grade III-IV		Any Chronic GVHD, NNST or NST		Chronic GVHD, NST	
	Yes	No	Yes	No	Yes	No	Yes	No
Median ATG clearance, L/kg/day	0.028	0.021	0.030	0.022	0.037	0.019	0.047	0.019
P value*	.030		.058		< .001		< .001	
Cutoff ATG level	>0.011				>0.029		>0.029	
Adjusted relative risk (95% CI)	9.4 (1.3-65.7)				2.9 (2.0-4.4)		5.5 (2.9-11)	
Adjusted *P* value†	.025				< .001		< .001	

* Mann-Whitney-Wilcoxon test (univariate analysis).
† Log-binomial regression (multivariate analysis).

Table 6
Correlation between Relative Decrease in ATG Level and Immune Cell Subset Counts

	Immune Cell Subset	R^*	*P* Value
Correlation between relative decrease in ATG level from day 0 to day 28 and cell count on day 28†	T cell	0.696	<.001
	B cell	0.134	NS
	NK cell	−0.197	NS

NS indicates not significant.
* Spearman's rank correlation coefficient.
† Relative decrease refers to the percentage decrease in ATG level from day 0 to day 28.

Remberger and Sundberg [2,3] found an association between high ATG levels on day 0 and low likelihood of aGVHD. However, there are some major differences between the 2 studies that might have influenced the results. Remberger and Sundberg included both bone marrow and PBSC recipients, whereas we included only PBSC recipients. The last dose of ATG was given on the day before graft infusion (day -1) to Remberger and Sundberg's patients, compared with the day of graft infusion (day 0) in our patients. Furthermore, Remberger and Sundberg used historical criteria for categorizing GVHD as acute (before day +100) or chronic (after day +100), whereas we used NIH criteria [20]. Thus, patients who developed NIH-defined cGVHD before day +100 were likely categorized in by Remberger and Sundberg as aGVHD, which theoretically could contribute to their finding of the association between day 0 ATG level and aGVHD (defined as pre–day +100 GVHD).

An important consideration is that Remberger and Sundberg measured total ATG, whereas we measured ATG capable of binding to lymphocytes. Thus, it is possible that the likelihood of developing aGVHD is reduced by antibodies within ATG that are capable of binding to cells other than lymphocytes, for example, to dendritic cells or endothelial cells, which are known to play an important role in the pathogenesis of aGVHD [29,30]. But if this is the case, then why was there a strong association between aGVHD and low levels of ATG capable of binding to lymphocytes on days +7 and +28? Given the association between the decrease in ATG level from day 0 to day +28 and aGVHD (Table 4), the correlation between the decreased ATG level and the day +28 T cell count (Table 5) and the association between high day +28 T cell count and aGVHD [31], it is conceivable that the low levels on day +7 and day +28 resulted from high T cell dose in the graft and/or expansion of alloreactive T cells between day 0 and days +7 and +28. These T cells could have adsorbed ATG, leading to decreased serum concentration. Thus, the low day +7 and day +28 ATG levels may be the result (and surrogate) of high T cell dose and/or alloreactive T cell expansion, rather than the cause of insufficient killing/inhibition of alloreactive T cells leading to aGVHD.

From a practical standpoint, for future studies of ATG levels as potential biomarkers predicting aGVHD, it may be important to measure ATG capable of binding to cells other than lymphocytes or to measure ATG capable of binding to lymphocytes after day 0.

Similar to Remberger and Sundberg [2] and Remberger et al. [32], we found no significant association between cell counts in grafts and a drop in ATG level. We also found no significant association between day +28 WBC or total lymphocyte count and the relative decrease (or clearance) in ATG level from day 0 to day +28. The lack of association could be related to the fact that on day +28, most WBCs are neutrophils and most lymphocytes are NK cells [28] rather than T cells that would be expected to adsorb ATG. The half-life of ATG calculated in our study is comparable to that in previous studies on clearance of ATG capable of binding to lymphocytes [12,19].

We found no significant association between ATG level at any of the 3 time points and overall survival, as represented by death and nonrelapse death. The association of ATG level with cGVHD is clinically significant, however, given that cGVHD is the most significant factor influencing the quality of life of transplantation survivors [33]. Thus, a lower incidence of cGVHD in the absence of increased incidence of relapse is expected to provide meaningful clinical benefit to transplant recipients.

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REFERENCES

1. Bacigalupo A, Lamparelli T, Bruzzi P, et al. Antithymocyte globulin for graft-versus-host disease prophylaxis in transplants from unrelated donors: 2 randomized studies from Gruppo Italiano Trapianti Midollo Osseo (GITMO). *Blood*. 2001;98:2942-2947.
2. Remberger M, Sundberg B. Rabbit-immunoglobulin G levels in patients receiving thymoglobulin as part of conditioning before unrelated donor stem cell transplantation. *Haematologica*. 2005;90:931-938.
3. Remberger M, Sundberg B. Low serum levels of total rabbit-IgG is associated with acute graft-versus-host disease after unrelated donor hematopoietic stem cell transplantation: results from a prospective study. *Biol Blood Marrow Transplant*. 2009;15:996-999.
4. Kim HJ, Min WS, Cho BS, et al. Successful prevention of acute graft-versus-host disease using low-dose antithymocyte globulin after mismatched, unrelated, hematopoietic stem cell transplantation for acute myelogenous leukemia. *Biol Blood Marrow Transplant*. 2009;15:704-717.
5. Bacigalupo A, Lamparelli T, Barisione G, et al. Thymoglobulin prevents chronic graft-versus-host disease, chronic lung dysfunction, and late transplant-related mortality: long-term follow-up of a randomized trial in patients undergoing unrelated donor transplantation. *Biol Blood Marrow Transplant*. 2006;12:560-565.
6. Bacigalupo A, Lamparelli T, Milone G, et al. Pre-emptive treatment of acute GVHD: a randomized multicenter trial of rabbit anti-thymocyte globulin, given on day +7 after alternative donor transplants. *Bone Marrow Transplant*. 2010;45:385-391.
7. Russell JA, Turner AR, Larratt L, et al. Adult recipients of matched related donor blood cell transplants given myeloablative regimens including pretransplant antithymocyte globulin have lower mortality related to graft-versus-host disease: a matched pair analysis. *Biol Blood Marrow Transplant*. 2007;13:299-306.
8. Bredeson CN, Zhang MJ, Agovi MA, et al. Outcomes following HSCT using fludarabine, busulfan, and thymoglobulin: a matched comparison to allogeneic transplants conditioned with busulfan and cyclophosphamide. *Biol Blood Marrow Transplant*. 2008;14:993-1003.
9. Finke J, Bethge WA, Schmoor C, et al. Standard graft-versus-host disease prophylaxis with or without anti–T-cell globulin in haematopoietic cell transplantation from matched unrelated donors: a randomised, open-label, multicentre phase 3 trial. *Lancet Oncol*. 2009;10:855-864.

10. Socie G, Schmoor C, Bethge WA, et al. Chronic graft-versus-host disease: long-term results from a randomized trial on graft-versus-host disease prophylaxis with or without anti-T-cell globulin ATG-Fresenius. *Blood*. 2011;117:6375-6382.
11. Mohty M, Labopin M, Balere ML, et al. Antithymocyte globulins and chronic graft-vs-host disease after myeloablative allogeneic stem cell transplantation from HLA-matched unrelated donors: a report from the Societe Francaise de Greffe de Moelle et de Therapie Cellulaire. *Leukemia*. 2010;24:1867-1874.
12. Waller EK, Langston AA, Lonial S, et al. Pharmacokinetics and pharmacodynamics of antithymocyte globulin in recipients of partially HLA-matched blood hematopoietic progenitor cell transplantation. *Biol Blood Marrow Transplant*. 2003;9:460-471.
13. Rebellato LM, Gross U, Verbanac KM, Thomas JM. A comprehensive definition of the major antibody specificities in polyclonal rabbit antithymocyte globulin. *Transplantation*. 1994;57:685-694.
14. Preville X, Flacher M, LeMauff B, et al. Mechanisms involved in antithymocyte globulin immunosuppressive activity in a nonhuman primate model. *Transplantation*. 2001;71:460-468.
15. Mohty M. Mechanisms of action of antithymocyte globulin: T-cell depletion and beyond. *Leukemia*. 2007;21:1387-1394.
16. Podgorny PJ, Ugarte-Torres A, Liu Y, et al. High rabbit-antihuman thymocyte globulin levels are associated with low likelihood of graft-vs-host disease and high likelihood of posttransplant lymphoproliferative disorder. *Biol Blood Marrow Transplant*. 2010;16:915-926.
17. Russell JA, Savoie ML, Balogh A, et al. Allogeneic transplantation for adult acute leukemia in first and second remission with a novel regimen incorporating daily intravenous busulfan, fludarabine, 400 CGy total body irradiation, and thymoglobulin. *Biol Blood Marrow Transplant*. 2007;13:814-821.
18. Hoegh-Petersen M, Roa L, Liu Y, et al. Low cytomegalovirus-specific T-cell counts at reactivation are associated with progression to high-level viremia or disease in seropositive recipients of hematopoietic cell grafts from seropositive but not seronegative donors. *Cytotherapy*. 2012;14:194-204.
19. Kakhniashvili I, Filicko J, Kraft WK, Flomenberg N. Heterogeneous clearance of antithymocyte globulin after $CD34^{+}$-selected allogeneic hematopoietic progenitor cell transplantation. *Biol Blood Marrow Transplant*. 2005;11:609-618.
20. Filipovich AH, Weisdorf D, Pavletic S, et al. National Institutes of Health Consensus Development Project on Criteria for Clinical Trials in Chronic Graft-versus-Host Disease, I: Diagnosis and Staging Working Group report. *Biol Blood Marrow Transplant*. 2005;11:945-956.
21. Przepiorka D, Weisdorf D, Martin P, et al. 1994 Consensus Conference on Acute GVHD Grading. *Bone Marrow Transplant*. 1995;15:825-828.
22. Lowe T, Bhatia S, Somlo G. Second malignancies after allogeneic hematopoietic cell transplantation. *Biol Blood Marrow Transplant*. 2007;13:1121-1134.
23. Landgren O, Gilbert ES, Rizzo JD, et al. Risk factors for lymphoproliferative disorders after allogeneic hematopoietic cell transplantation. *Blood*. 2009;113:4992-5001.
24. LaCorcia G, Swistak M, Lawendowski C, et al. Polyclonal rabbit antithymocyte globulin exhibits consistent immunosuppressive capabilities beyond cell depletion. *Transplantation*. 2009;87:966-974.
25. Popow I, Leitner J, Majdic O, et al. Assessment of batch to batch variation in polyclonal antithymocyte globulin preparations. *Transplantation*. 2012;93:32-40.
26. Zou G. A modified poisson regression approach to prospective studies with binary data. *Am J Epidemiol*. 2004;159:702-706.
27. Toutain PL, Bousquet-Melou A. Plasma clearance. *J Vet Pharmacol Ther*. 2004;27:415-425.
28. Bosch M, Dhadda M, Hoegh-Petersen M, et al. Immune reconstitution after antithymocyte globulin–conditioned hematopoietic cell transplantation. *Cytotherapy*. 2012;14:1258-1275.
29. Shlomchik WD, Couzens MS, Tang CB, et al. Prevention of graft-versus-host disease by inactivation of host antigen-presenting cells. *Science*. 1999;285:412-415.
30. Korngold R, Sprent J. Negative selection of T cells causing lethal graft-versus-host disease across minor histocompatibility barriers: role of the H-2 complex. *J Exp Med*. 1980;151:1114-1124.
31. Podgorny PJ, Liu Y, Dharmani-Khan P, et al. Immune cell subset counts associated with graft-versus-host disease. *Biol Blood Marrow Transplant*. 2014;20:450-462.
32. Remberger M, Svahn BM, Mattsson J, Ringden O. Dose study of thymoglobulin during conditioning for unrelated donor allogeneic stem-cell transplantation. *Transplantation*. 2004;78:122-127.
33. Joseph RW, Couriel DR, Komanduri KV. Chronic graft-versus-host disease after allogeneic stem cell transplantation: challenges in prevention, science, and supportive care. *J Support Oncol*. 2008;6:361-372.